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(54) Title: BISPECIFIC REAGENTS FOR REDIRECTED TARGETING OF LOW DENSITY LIPOPROTEIN

(57) Abstract

Bispecific molecules which react both with an Fcγ receptor for immunoglobulin G (IgG) of human effector cells and with human low density lipoprotein (LDL), or fragment thereof, are disclosed. The bispecific molecules bind to an Fcγ receptor without being blocked by the binding of IgG to the same receptor. The bispecific molecules are useful for targeting human effector cells for degradation of LDL *in vivo*. Also disclosed are methods of treating atherosclerosis using these bispecific molecules.

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BISPECIFIC REAGENTS FOR REDIRECTED TARGETING OF LOW DENSITY LIPOPROTEIN

Background of the Invention

Atheromatous lesions consist of numerous cellular and acellular elements. Macrophages are a major constituent of these lesions as they develop into characteristic cholesteryl ester-laden foam cells (Ross, R. (1986) New England J. Med. 314:488-500; Munro, J.M. and Cotran, R.S. (1988) Laboratory Invest. 58:249-261). For macrophages, foam cell development during atherogenesis is ultimately dependent upon uptake of various forms of low density lipoprotein (LDL) (reviewed in Brown, M.S. and Goldstein, J.L. Ann. Rev. Biochem. 52:223-261). Of late, emphasis has been placed on the importance of the interaction of chemically modified or oxidized LDL with macrophage scavenger receptors (reviewed in Steinberg, D., et al. (1988) New England J. Med. 320:915-924), which may occur during atherogenesis in vivo (Steinberg, D., et al. (1988) New England J. Med. 320:915-924; Haberland, M.E., et al. (1988) Science 241:215-218; Palinski, W., et al. (1989) Proc. Natl. Acac. Sci. USA 86:1372-1376; Rosenfeld, M.E., et al. (1990) Arteriosclerosis 10:336-349; Boyd, H.C., et al. (1990) Am. J. Path. 135:815-825).

Foam cell development may also be influenced by the interaction of lipoproteins with pathways other than those associated with scavenger receptors. In particular, recent studies have shown that immune complexes consisting of LDL bound to anti-LDL antibodies (LDL-IC) can cause foam cell development in vitro through interaction with IgG Fc receptors (FcγR) when administered to both mouse (Klimov, A.N., et al. (1985) Atherosclerosis 58:1-15) and human macrophages (Lopes-Virellâ, M.F., et al. (1991) Arteriosclerosis and Thrombosis 11:1356-1367; Griffth, R.L., et al. (1988) J. Exp. Med. 168:1041-1059). LDL-IC (reviewed in Orekhov, A.N (1991) Curr. Opin. Lipidology 2:329-333) consisting of antibodies bound to either native or oxidized LDL exist in numerous situations in vivo (Szondy, E., et al. (1983) Atherosclerosis 49:69-77; Parums, D.V., et al. (1990) Arch. Pathol. Lab. Med. 114:383-387; Beaumont, J.L., et al. (1988) Atherosclerosis 74:191-201; Kigore, L.L., et al. (1985) J. Clin. Invest. 76:225-232), and in several cases have been correlated with abnormalities of lipid metabolism and atherosclerosis (Szondy, E., et al., supra; Parums, D.V., et al., supra; Beaumont, J.L., et al., supra; Kigore, L.L., et al., supra; Cohen, L., et al. (1966) Am. J. Med. 40:299-316).

Human Fcγ receptors (FcγR) (reviewed in Fanger, M.W., et al. (1989) Immunology Today 10:92-99), of which there are three structurally and functionally distinct types (i.e., FcγRI, FcγRII and FcγRIII), are well-characterized cell surface glycoproteins that mediate phagocytosis or antibody-dependent cell cytotoxicity (ADCC) of immunoglobulin G (IgG) opsonized targets.

Bispecific antibody technology has been used to evaluate the function of specific FcγR. Investigators have shown that FcγR are the only cell surface molecules on myeloid cells capable of triggering phagocytic or cytotoxic function (Shen, L., et al. (1986) J. Immunol. 137:3378-3382; Shen, L. et al. (1987) J. Immunol. 139:534-538; Connor, R.I., et al. (1990) J. Immunol. 145:1483-1489; Anderson, C.L., et al. (1990) J. Exp. Med. 171:1333-1345). However, clear differences in the functional ability of the different FcγR could be demonstrated that was dependent not only on the FcγR class or isoform but on the state of activation and differentiation of the cell (Fanger, M.W., et al. (1989) Immunology Today 10:92-99; Van de Winkel, J.G. and Anderson, C.L. (1991) J. Leukocyte Biol. 49:511-524).

Summary of the Invention

This invention pertains to bispecific molecules which can bind human low density lipoprotein (LDL) and/or which can simultaneously target human LDL for ingestion and metabolic degradation by effector cells such as monocytes, macrophages, eosinophils, granulocytes, platelets and neutrophils. The bispecific molecules of this invention have a first binding specificity for human LDL and a second binding specificity for an FcγR receptor for immunoglobulin G (IgG), such as the human FcγR receptors FcγRI, FcγRII and FcγRIII. In a preferred embodiment, such bispecific molecules are capable of binding to IgG-occupied Fcq receptors on effector cells. For specifically targeting FcγRI, it is preferred that the bispecific molecule have a binding specificity for an epitope on the receptor which is distinct from the Fc ligand binding domain of the receptor for the Fc region of IgG.

Bispecific molecules of the invention can be a bispecific antibody (i.e., a single antibody or antibody fragment with a dual binding specificity), a heteroantibody (i.e., an aggregate of two or more antibodies or antibody fragments, each having a different binding specificity) or a single chain bispecific polypeptide. In general, a bispecific antibody, heteroantibody or single-chain bispecific polypeptide comprises: at least one antigen binding region derived from an anti-Fc receptor antibody whose binding to human Fc receptor is not blocked by human IgG; and at least one antigen binding region specific for human LDL.

The binding of a bispecific molecule of the present invention to an appropriate effector cell results in a targeted effector cell, i.e., an effector cell to which is bound a bispecific antibody or heteroantibody containing antigen binding regions specific for human LDL. The targeted effector cells can be used to bring about phagocytosis of LDL by the effector cells.

The bispecific molecules of this invention have therapeutic as well as diagnostic applications. As a therapeutic agent, the bispecific molecules can be administered to an individual alone, or pre-bound to effector cells having the appropriate Fc receptor prior

to administration. In either form, the bispecific molecules are administered in an amount sufficient to induce phagocytosis of human LDL to thereby reduce LDL levels in the individual. Bispecific molecules of the invention can also be used in conjunction with other molecules such as cytokines (e.g., interferon-γ) which can activate or enhance their therapeutic potential.

As a diagnostic agent, the bispecific molecules can be cultured ex vivo with an individual's white blood cells (e.g., monocytes or macrophages), to evaluate the capacity or tendency of such cells to accumulate cholesteryl ester and develop into foam cells. The tendency of white blood cells of the individual to develop into foam cells indicates that the individual is at risk of developing atherosclerosis.

Brief Description of the Drawings

FIG. 1 is a graphic representation of the binding of ^{125}I-LDL to human monocyte receptors mediated through bispecific molecules. Monocytes (A) or polymorphonuclear cells (PMN) (B) were treated with 25μg/ml of the indicated bispecific antibody and 100μg/ml of ^{125}I-LDL plus equivalent amounts of anti-LDL alone. To show specificity, the binding of bispecific antibodies was also done in the presence of an excess amount (0.5 mg/ml) of the corresponding unconjugated anti-FcγR or anti-HLA Class I antibody (hatched bars). Shown are the means ± SD of triplicate measurements of ng ^{125}I-LDL bound per mg cell protein.

FIG. 2 is a graphic representation of the effects of bispecific LDL immune complexes (LDL*-IC) on myeloid cells. Monocytes (A) in 10% fetal calf serum (FCS) were treated with 4 μg/ml BODIPY-LDL plus varying amounts of anti-FcγRI x anti-LDL (circles), anti-FcγRII x anti-LDL (triangles), or anti-HLA Class I x anti-LDL (squares), for 2 hours and analyzed by flow cytometry as described in the examples. In (B), monocytes were treated similarly with BODIPY-LDL plus anti-FcγRII x anti-LDL at 4°C (triangles) and 37°C (circles). Shown in Fig. 2B are the means ± SD of triplicate measurements of cell-associated LDL*.

FIG. 3 is a graphic representation of the accumulation of bispecific LDL*-IC with time of culture at 37°C. Monocytes in 10% FCS were treated at 37°C for the indicated times with BODIPY-LDL alone or bispecific LDL*-IC consisting of 0.5 μg/ml of anti-FcγRII x anti-LDL plus 1.33 μg/ml of BODIPY-LDL as described in the examples. Cell-associated fluorescence was analyzed by flow cytometry. Shown are the means ± SD of triplicate measurements of cell-associated fluorescence.

FIG. 4 is a graphic representation of degradation of ^{125}I-LDL by human monocytes mediated through FcγR. Monocytes in 10% LPDS were treated for four hours at 37°C with ^{125}I-LDL plus saturating amounts of bispecific anti-FcγRI x anti-LDL, anti-FcγRII x anti-LDL, or anti-FcγRIII x anti-LDL. Controls are shown consisting of ^{125}I-LDL in the absence of antibody, and ^{125}I-LDL plus an equivalent

amount of unconjugated anti-LDL. The cell supernatants were analyzed for acid-soluble products that did not contain free iodide. All values were corrected for spontaneous degradation by subtracting the value of the appropriate cell-free control. Shown are the means ± SEM of duplicate measurements from three experiments. "*", significant to both controls; "**", significant to anti-LDL control only.

Detailed Description of the Invention

There are three structurally and functionally distinct types of human FcγR receptors, FcγRI, FcγRII and FcγRIII. FcγRI is a 70 kDa glycoprotein which binds to monovalent IgG with high affinity, and an equilibrium dissociation constant(Kd) of 10^{-8}-10^{-9} M for human IgG1 and IgG3, and mouse IgG2a and IgG3 (Kurlander et al. (1982) J. Clin. Invest. 69:1-8; Jones et al. (1985) J. Immunol. 135:3348-3353). Two different epitopes on FcγRI, each distinct from the ligand-binding site, have been defined using mAb 32 and mAb 22, (Guyre, P.M., et al., submitted) and the binding of these mAbs to FcγRI is not blocked by human IgG or by immune complexes.

FcγRII (CDw32) is a 40 kDa glycoprotein (Anderson, C.L. (1982) J. Exp. Med. 156:1794-1806). Two forms of FcγRII were identified based on functional assays and biochemical analysis (Anderson, C.L. and Looney, R.J. (1986) Immunol. Today 7:264-266). Recent cDNA cloning experiments support the concept of multiple receptor subtypes, as cDNAs have been isolated that identify at least three related molecules possessing identical extracellular, but different cytoplasmic domains (Mellman, I. (1988) Current Opin. Immunol. 1:16-25; Stengelin, S., et al. (1988) EMBO J. 17:1053-1059). All forms of FcγRII have low affinity for monovalent human IgG1, and thus appear to be specific for immune complexes and opsonized particles.

FcγRIII (CD16) also has low affinity for monomeric IgG, and was originally identified as a 50-70 kDa glycoprotein. The cDNA for FcγRIII encodes a predicted peptide of 233 residues (Mr 26 kDa) which, on polymorphonuclear neutrophils (PMNs), appears to be bound to the membrane through a phosphatidylinositol glycan (PIG) linkage (Selvaraj, P., et al. (1988) Nature 333:565-567; Simmons, D. and Seed, B. (1988) Nature 333:568-570). Several lines of evidence suggest that PMNs express a different form of FcγRIII than macrophages and natural killer (NK) cells. In addition to the differences between FcγRIII on granulocytes and NK cells, a structural polymorphism of this receptor on neutrophils has been demonstrated that may result from two allotypic forms of FcγRIII (Wemer, G., et al. (1986) in Leukocyte Typing II (vol. 3) (Reinherz E.L. et al. ed.) pp. 109-121 Springer Verlag).

The bispecific molecules of the invention have at least two distinct binding specificities: a binding specificity for human low density lipoprotein (LDL); and a binding specificity for an Fc-receptor for immunoglobulin G (IgG) of a human effector cell.

In the preferred embodiment, bispecific molecules of the invention bind a human Fc receptor without being blocked by the binding of human IgG to the receptor. Since the FcγRI receptor binds monomeric IgG with high affinity, it is preferred that the receptor binding specificity is provided by a binding agent which binds to an epitope of the FcγRI receptor, which is distinct from the Fc (or ligand binding) site of the receptor. The preferred Fcγ receptor binding agent is an antibody, antibody fragment, antibody variable region, or genetic construct having the following characteristics: (a) the agent reacts specifically with human FcγRI receptor, FcγRII receptor or FcγRIII receptor; (b) the agent reacts with human FcγRI receptor, FcγRII receptor or FcγRIII receptor through the agent's antigen binding region and not its Fc portion; (c) the agent reacts with an epitope of human FcγRI receptor, FcγRII receptor or FcγRIII receptor which is distinct from the Fc binding (i.e., ligand binding) site of the receptor; and (d) the agent binds ligand (e.g., Fc)-occupied receptor.

The anti-Fcγ receptor antibodies of this invention can be produced as described in U.S. Patent No. 4,954,617 (Fanger et al., "Monoclonal Antibodies to Fc Receptors for Immunoglobulin G on Human Mononuclear Phagocytes"), the contents of which are incorporated herein by reference.

The binding specificity for human LDL can be provided by a binding agent which specifically binds an epitope of human LDL. For example, anti-LDL antibodies or LDL-binding fragments thereof can be used. These antibodies can be produced by conventional monoclonal antibody methodology, e.g., the standard somatic cell hybridization technique of Kohler et al. (Nature (1975) 256:495), using human LDL, or fragment thereof, as the immunogen. In brief, an animal such as a mouse is immunized with human LDL, or portion thereof. The human LDL can be purified or partially purified from serum, or produced by chemical synthesis or recombinant technique. Human LDL can be purified from serum, chemical precursors or cellular material by techniques known in the art including ion-exchange chromatography, gel filtration chromatography, electrophoresis or immunopurification using an antibody specific for human LDL or portion thereof. After immunization, B cells are taken from the immunized animal and fused with an immortalizing cell such as a myeloma cell. See, e.g., Fung et al. (1987) Biotechnol. 5:940-946. It will be appreciated that fragments of human LDL can also be employed as the component to which a binding specificity is provided. A preferred anti-LDL monoclonal antibody is A01609, which is a high affinity IgG1 ($K_a = 5 \times 10^{10}$/mol) antibody that recognizes an epitope on human apoprotein B, with no cross-reactivity to apoprotein A-I or A-II (Medix Biotech, Inc., Foster City, CA).

Preferred bispecific molecules of the invention are bispecific antibodies, heteroantibodies and single-chain bispecific polypeptides. Bispecific antibodies resemble single antibodies (or antibody fragments) which have two different antigen

binding regions (variable regions). Bispecific antibodies of this invention have one binding region for a human Fcγ receptor (i.e., FcγRI, FcγRII, FcγRIII) and one binding region for an epitope of human LDL. Bispecific antibodies can be produced by chemical techniques (see e.g., Kranz et al. Proc. Natl. Acad. Sci. USA 78:5807 (1981)), by "polydoma" techniques (see U.S. Patent 4,474,893, issued to Reading), or by recombinant DNA techniques.

Heteroantibodies are two or more antibodies, or antibody binding fragments (Fab) linked together, each antibody or fragment having a different binding specificity. Heteroantibodies of the invention comprise an antibody (or antigen binding fragment) specific for a human Fcγ receptor (i.e., FcγRI, FcγRII, FcγRIII), coupled to an antibody (or antigen binding fragment) specific for an epitope of human LDL. Heteroantibodies can be prepared by conjugating an Fcγ receptor antibody with an antibody specific for an epitope of human LDL. A variety of coupling or crosslinking agents can be used to conjugate the antibodies including protein A, carboiimide, dimaleimide, dithio-bis-nitrobenzoic acid (DTNB), N-succinimidyl-S acetyl-thioacetate (SATA), and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). SATA, DTNB and SPDP are preferred coupling agents. Procedures for crosslinking antibodies with these agents are well-known in the art. See e.g., Karpovsky et al. (1984) J. Exp. Med. 160:1686; Liu et al. (1985) Proc. Natl. Acad. Sci. USA 82:8648; Segal et al., U.S. Patent No. 4,676,980 (June 30, 1987); and Brennan Biotech. 4:424 (1986).

Bispecific molecules of this invention can be prepared by conjugating a gene encoding a binding specificity for human LDL (i.e., an epitope of human LDL) to a gene encoding at least the binding region of an antibody chain which recognizes a human Fcγ receptor (i.e., FcγRI, FcγRII, FcγRIII). This construct is transfected into a host cell (such as a myeloma) which constitutively expresses the corresponding heavy or light chain, thereby enabling the reconstitution of a bispecific, single-chain antibody, two-chain antibody (or single chain or two-chain fragment thereof such as Fab) having a binding specificity for human LDL and for a human FcγR. Construction and cloning of such a gene construct can be performed by standard procedures.

The function of human Fcγ receptors is dictated in part by the type of effecor cell on which they are displayed. Effector cells which can be targeted by the bispecific molecules of the invention include monocytes, macrophages, leukocytes, activated neutrophils, activated natural killer (NK) cells, tissue macrophages, platelets and eosinophils. Of particular interest as targets due to their role in the pathogenesis of atherosclerosis are human monocytes and macrophages which are capable of developing into foam cells (i.e., cholesteryl ester-laden white blood cells) which are a major constituent of atheromatous lesions.

Many factors influence the number of receptors expressed and detected on effector cells. For example, cell culture conditions, the presence or absence of human

IgG which may interfere with measurement, natural cytokines to which the cells were exposed in vivo, and hormones and cytokines in cell culture medium may influence receptor expression. FcγRI expression is almost exclusively restricted to mononuclear phagocytes whereas FcγRII is expressed on the surface of virtually all hematopoietic cells except erythrocytes and is probably the sole FcγR on human platelets (Rosenfeld, S.I., et al. (1985) J. Clin. Invest. 76:2317-2322). FcγRIII is widely recognized for its high level of expression (100,000 - 200,000 sites per cell) on human neutrophils (Fleit, H.B., et al. (1982) PNAS USA 79:3275-3279; Petroni, K.C., et al. (1988) J. Immunol. 140:3467-3472).

Myeloid cells, with the exception of eosinophils, when exposed to interferon γ (IFN-γ) have been shown to increase expression of FcγRI and increase killing of red blood cells via FcγRI, FcγRII and FcγRIII (see e.g., Fanger, M.W., et al. (1989) Immunol. Today 10:92-99). Effector cells can also be activated by other cytokines such as tumor necrosis factor (TFN), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), lymphotoxin, interleukin-2 (IL-2) and interleukin-3 (IL-3).

As a therapeutic agent, bispecific molecules of this invention can be administered to an individual to target human LDL for phagocytosis and subsequent degradation by an effector cell, such as a human monocyte or macrophage. To influence lipoprotein metabolism in vivo, the bispecific molecules can be targeted to an Fcγ receptor which is not involved in macrophage foam cell development during atherogenesis. For example, a therapeutic amount of a heteroantibody comprising a first antibody or antigen-binding fragment thereof which immunologically binds human LDL, and a second antibody or antigen-binding fragment thereof which immunologically binds an Fcγ receptor can be administered to an individual in the form of a physiologically acceptable solution. The heteroantibody will target LDL to the Fcγ receptor on the monocyte or macrophage to result in LDL uptake and metabolism such that cholesteryl ester accumulation is reduced.

Alternatively, effector cells from the individual can be obtained from the host to be treated or any other immunologically compatible donor and cultured in vitro with bispecific molecules to produce opsonized effector cells having attached bispecific molecules. The opsonized effector cells can be administered as a suspension of cells in a physiologically acceptable solution. The number of cells administered can be in the order of 10^8-10^9, but will vary depending on the therapeutic purpose. Opsonized effector cells or bispecific molecules can be administered to an individual intravenously, intramuscularly, or intraperitoneally at dosages and for lengths of time known in the art, (see e.g., Ball, E.D., et al. J. Hemototherapy (1992) in press; de Leij, L., et al. (Oct. 9-13. 1990) Second International Conference, Seillac, France. Bispecific Antibodies and Targeted Cellular Cytotoxicity, pp. 249-253; Clark, M., et al.

(Oct. 9-13, 1990) Second International Conference, Seillac, France, Bispecific Antibodies and Targeted Cellular Cytotoxicity, pp. 243-247; and Nitta, T. et al. (Feb. 17, 1990) The Lancer 335:368-371).

The bispecific molecules of this invention are particularly well suited for in vivo therapy even when a large excess of human IgG is present in vivo ("antibody blockade"). The anti-FcγR component of the bispecific molecules bind epitopes of the appropriate Fc γR distinct from the ligand IgG binding domain which are not blocked by endogenous IgG. Thus, IgG binding to the receptor does not interfere with binding of the bispecific molecule to an effector cell or interfere with functioning of effector cells.

The bispecific molecules of the invention can also be used in diagnostic applications. Recent studies indicate that LDL immune complexes consisting of anti-LDL antibodies bound to human LDL may contribute to macrophage foam cell development by uptake through IgG Fc receptors and/or macrophage scavenger receptors. As human mononuclear phagocytes possess all three classes of IgG Fc receptors, FcγRI, FcγRII and FcγRIII, the effects of LDL immune complexes can be determined with respect to each type of receptor. Bispecific molecules of the invention comprising an anti-Fcγ receptor antibody linked to an anti-LDL antibody can be used to target a specific Fcγ receptor to determine which receptors may be involved in the development of cholesteryl ester-laden foam cells during atherogenesis. Fcγ receptors involved in macrophage foam cell development can be interefered with, blocked or down-regulated to reduce the formation of atheromatous lesions. For example, an anti-Fcγ receptor antibody can be used to block binding of the receptor. Fcγ receptors which are not involved in foam cell development can be targeted with bispecific molecules of the invention to facilitate macrophage phagocytosis and degradation of LDL.

To diagnose an individuals's risk of developing atherosclerosis, white blood cells, such as monocytes or macrophages can be obtained from the individual and contacted with bispecific molecules of the invention and LDL or LDL immune complexes under conditions appropriate for binding of the bispecific molecule to the Fcq receptor and LDL or LDL immune complexes. The level of cholesteryl ester accumulated by the cell can be determined as indicative of the tendency of the cell to develop into a foam cell and identifies the individual as at risk of developing atherosclerosis in the context of immune complex stimulation. Such a determination may also indicate the tendency of the cell to metabolize LDL and accumulate cholesteryl ester via other pathways, such as those associated with scavenger receptors.

The following examples describe the effects of bispecific LDL immune complexes directed to Fcγ receptor types I, II, and III on monocytes in comparison to the effects of similarly prepared bispecific complexes that targeted LDL to HLA Class I antigens. Each type of bispecific molecule was effective in targeting ^{125}I-LDL to its respective site on the cell surface. Using fluorophore-labeled LDL and flow cytometry.

bispecific complexes directed to Fcγ receptor types I or II, but not to HLA Class I antigens, caused a two to seven-fold increase in cell-associated fluorescence relative to control cells treated with LDL in the absence of heteroantibody. Using ^{125}I-labeled complexes, metabolic degradation of LDL was demonstrated in association with each of the three types of Fcγ receptors.

The invention is illustrated further by the following examples.

METHODS

Chemicals and reagents

SATA and SPDP, (N-succinimidyl-S-acetylthioacetate and N-succinimidyl-3-[2-pyridyldithio]propionate, respectively), dimethylformamide, and hydroxylamine were obtained from Pierce, Rockford, IL. Unless otherwise indicated, all other reagents were obtained locally or from Sigma Chemical Co., St. Louis, MO.

Monoclonal antibodies (mAb)

All mAb used were of murine origin. Purified anti-FcγRI (mAb 22, an IgG1), anti-FcγRII (mAb IV.3, an IgG2b), and anti-FcγRIII (mAb 3G8, an IgG1) were obtained from Medarex, Inc., W. Lebanon, NH. Anti-LDL mAb A01609 is a high affinity IgG1 (K_a=5 x 10^{10}/mol) that recognizes an epitope on human apoprotein B, with no cross-reactivity to apo A-I or A-II and was obtained from Medix Biotech, Inc., Foster City, CA. This mAb reacted to adsorbed native LDL by ELISA, at 4°C and 37°C, under conditions that inhibit oxidation (Palinski, W., et al. (1990) Arteriosclerosis 10:325-335). Anti-HLA Class I (mAb BBM.1, an IgG2b), that recognizes an epitope on β_2-microglobulin (Brodsky, F.M., et al. (1979) Eur. J. Immunol. 9:536-545), was obtained from American Type Culture Collection, Rockville, MD.

Production of bispecific antibodies

Unless otherwise indicated, all steps were carried out at room temperature. Intact mAb were used for all conjugations except for preparation of anti-FcγRIII x anti-LDL, which was done with F(ab')$_2$ fragments. Prior to derivatization, each mAb was dialyzed into Ca^{2+}, and Mg^{2+}-free phosphate-buffered saline (PBS), pH 7.5, containing 1.25 mM EDTA and used at approximately 2-6 mg/ml. Heteroantibodies were prepared by first reacting, anti-myeloid cell mAb with the heterobifunctional crosslinker SPDP (Karpovsky, B., et al. (1984) J. Exp. Med. 160:1686-1701), and anti-LDL mAb with SATA (Duncan, R.J.S., et al. (1983) Anal. Biochem. 132:68-73). To prepare SPDP-labeled mAb (anti-FcγR or anti-HLA class 1), SPDP was added to mAb at an 8:1 molar ratio (from an SPDP stock solution of 3.2 mg/ml freshly prepared in ethanol) for one

hour under N_2. SATA-anti-LDL was prepared by adding SATA to anti-LDL mAb at a 12:1 molar ratio (freshly prepared in dimethylformamide, 2% v/v added to mAb) for one hour also under N_2. Both preparations were dialyzed overnight into 50 mM sodium phosphate plus 5 mM EDTA, pH 7.5, at 4°C. The next day SPDP-mAb and SATA-anti-LDL were combined at a 2:1 molar ratio respectively, and coupling was initiated by addition of freshly-prepared hydroxylamine (added from a 0.5M 10X stock) in 50 mM sodium phosphate plus EDTA, pH 7.5. Coupling was allowed to continue overnight at room temperature under N_2. After coupling, the heteroantibodies were separated from unreacted monomers by HPLC on TSK-250 or GPC 300 analytical sizing columns (SynChron, Inc., Lafayette, IN) equilibrated in endotoxin-free PBS. Pooled fractions were subsequently rechecked by HPLC to verify the absence of IgG monomers. With this protocol, approximately 50% of the total protein typically is recovered as heteroantibody. All fractions were sterilized by passage through a 0.2 μm filter, and stored at 4°C. Protein content was determined by BCA assay (Pierce) and by determination of optical density (A_{280}) using an IgG extinction coefficient of 1.43 OD/mg/ml.

Lipoproteins

LDL (d > 1.019 < 1.063 g/ml) was isolated from the serum of fasted donors by ultracentrifugation according to standard techniques (Goldstein, J.L., et al. (1983) Methods in Enzymology 98:241-260). A cocktail of freshly prepared protease inhibitors (Cadigan, K.M., et al. (1988) J. Biol. Chem. 263:274-282) in addition to EDTA was added to the blood at the outset. Following isolation, LDL was dialyzed extensively into buffer containing, 10 mM sodium phosphate, 1.2 mM EDTA, and 140 mM NaCl, pH 7.4, prepared in sterile pyrogen-free water. Final storage was in the presence of 25 μ g/ml gentamicin under N_2 (Lopes-Virella, M.F., et al., supra). Protein content was determined by a modified Lowry assay using bovine serum albumin (BSA) as the standard (Peterson, G.L.A. Anal. Biochem. 83:346-356). LDL was labeled with ^{125}I according to the McFarlane method as described (Goldstein, J.L., et al., supra). The specific activity of all preparations ranged from 220-440 CPM per ng protein, and all preparations were used within three weeks. BODIPY-LDL (LDL*) and BODIPY-acetyl-LDL (AcLDL*), conjugates with excitation and emission spectra similar to that of fluorescein, were obtained from Molecular Probes, Inc., Eugene, OR and used within three weeks of delivery. AcLDL was prepared by the method of Basu et al. (Basu, S.K., et al. (1976) Proc. Natl. Acad. Sci. USA 73:3178-3182); in preliminary experiments all AcLDL preparations caused greater than 80% inhibition of uptake of AcLDL* by monocytes at 37°C; likewise, all LDL preparations caused geater than 80% inhibition of uptake of LDL*. Lipoprotein deficient serum was prepared from bovine calf serum by ultracentrifugation according to standard techniques (Goldstein, J.L., et al., supra). It

was adjusted to 5 mg/ml protein with saline, sterilized by passage through a 0.2 μm filter, and used in RPMI 1640 at 10%.

Preparation of Leukocytes

Freshly isolated monocytes or polymorphonuclear (PMN) were used in all experiments. Monocytes were obtained from leukapheresis packs of normal donors as described previously (Shen, L., et al. (1986) Clin. Exp. Immunol. 65:387-395). Platelets were removed by three washes in Versene buffer (Sporn, S.A., et al. (1990) J. Immunol. 144:4434-4441) (Gibco, Grand Island, NY), and final preparations typically were geater than 90% monocytes as judged by morphology. In all experiments, cells were washed extensively in RPMI 1640 (JRH Biologicals, Inc., Lenexa, KS) containing 2 mg/ml BSA, 15 mM HEPES, and 25 μg/ml gentamicin (monocyte culture medium), and resuspended in RPMI 1640 plus 10% FCS and gentamicin unless otherwise indicated. PMN were separated from whole venous blood of normal donors by the discontinuous Percoll gradient procedure (Haslett, C., et al. (1985) Am. J. Pathol. 119:101-110). Final suspensions were in RPMI 1640 plus 10% FCS and gentamicin unless otherwise indicated. Viability, as assessed by exclusion of ethidium bromide (Mishell, B.B. and Shiigi, S.M. (1980) Selected Methods in Immunology, pp. 21-22), was always greater than 95% for both monocytes and PMN.

125I-LDL Binding Assay

Binding of ^{125}I-LDL to monocytes in the presence and absence of heteroantibodies was done on ice in a two step procedure. 1 x 10^6 monocytes in RPMI 1640 plus 10% LPDS were added to eppendorf tubes containing saturating amounts of heteroantibodies for 1 hour on ice. In specificity experiments, binding of heteroantibodies was done in the presence of 0.5 mg/ml of the corresponding unconjugated anti-FcγR or anti-HLA Class I antibodies. After binding, the cells were washed twice in one ml of ice cold 10% LPDS, and then received a saturating amount of ^{125}I-LDL (100 mg/ml) in 10% LPDS, also for one hour on ice. In some experiments, the latter step was also done in the presence of 2 mg/ml of unlabeled LDL, as a control for LDL specificity. The cells were then washed once in ice cold PBS containing 2 mg/ml of BSA, followed by two washes in PBS containing no BSA. Suspensions of cells were pelleted through 200 μl of 170,000 kDa dextran in PBS in a microfuge, and cell pellets were cut from the tubes and counted in a gamma counter. Some pellets were lysed in 0.1 N NaOH for determination of total cell protein by modified Lowry assay (Peterson, G.L.A., supra). Results are expressed as ng ^{125}I-LDL bound per mg cell protein.

Uptake of bispecific LDL immune complexes

All experiments were done under sterile conditions. For each experiment, bispecific LDL immune complexes (bispecific LDL*-IC) were freshly prepared by combining 4 μg/ml of LDL* with heteroantibodies in eppendorf tubes in monocyte culture medium (without serum or unlabeled LDL unless otherwise indicated), and incubating for one hour at room temperature, prior to adding cells in RPMI 1640 plus 10% FCS. That concentration of LDL* was chosen because it gave a satisfactory signal to noise ratio. In all experiments, a control consisting of LDL* alone was set up by substituting medium for heteroantibody. The final volume of bispecific complexes prior to addition of cells was 50 ml; 100 ml of cells at 1 x 10^7/ml were added for a final volume of 150 ml. The tubes were then mixed by gentle pipetting, and incubated at 4°C or 37°C as indicated. Final mAb or heteroantibody concentrations are shown in each figure; the final concentration of LDL* in all preparations was 1.33 μg/ml unless otherwise indicated. Blocking studies with unlabeled LDL or unlabeled AcLDL were done by pretreating cells with an excess amount of lipoprotein for one hour at room temperature, and then adding them to bispecific complexes without washout.

Analysis of cell-associated fluorescence of LDL* and AcLDL*

In all experiments, cells were removed after various times of incubation and washed twice with one ml of PBS at room temperature. The final suspension was in cold 1% methanol-free paraformaldehyde (Eastman-Kodak, Rochester, NY) in PBS. For cytofluorogaphic analysis, samples were analyzed for green fluorescence on a FACScan (Becton-Dickinson). The excitation wavelength was 488 nm and emission at 515 nm was analyzed. Monocyte or PMN were rated on the basis of blue forward vs. right angle light scatter. In most experiments, the gain for linear fluorescence was set such that the LDL*-alone or AcLDL*-alone control had a mean fluorescence intensity of approximately 100 on a scale of 1000; for cells treated with LDL*-alone a second region was also set arbitrarily such that 5% of the total population was positive for green fluorescence.

LDL degradation assay

The degradation of bispecific LDL-IC following uptake through FcγR was assessed by a standard type assay of acid-soluble products that did not contain free iodide (Goldstein, J.L., et al., supra). All treatments were corrected for background degradation by including cell-free controls, with and without antibody. For these studies monocytes were treated with and without saturating amounts of heteroantibody for one hour on ice; all tubes then received 100 μg/ml of ^{125}I-LDL in 10% LPDS. The cells and cell-free controls were incubated for 4 hours at 37°, after which cell supernatants were harvested and analyzed for acid-soluble products (Goldstein, J.L., et al., supra). Cell

pellets were immediately chilled on ice, washed, spun through dextran and counted in a gamma counter as described above. In some experiments, parallel sets of cells were kept on ice and processed for determination of cell-bound radioactivity as described above. In all experiments cell pellets were lysed in 0.1N NAOH for determination of total protein by modified Lowry assay (Peterson, G.L.A., supra). Results from degradation assays are expressed as ng ^{125}I-LDL degraded per mg cell protein per four hours incubation at 37°C.

Statistics

Groups of data from three degradation assays were compared by one-way analysis of variance, and multiple comparisons were made with the Neuman-Keuls test. P values = to 0.05 were taken to indicate significance (Zar, J.H. (1974) Biostatistical Analysis, pp. 185-191).

RESULTS

Binding of 125I-LDL to cells mediated through FcγR

To determine the capacity and specificity of heteroantibodies to deliver LDL to cells via FcγR, monocytes on ice were treated with 100 µg/ml of ^{125}I-LDL plus saturating amounts of the heteroantibodies anti-FcγRI x anti-LDL, anti-FcγRII x anti-LDL, or anti-HLA Class I x anti-LDL as described in "Methods". PMN were used to test anti-FcγRIII x anti-LDL, since they express much greater amounts of FcγRIII than monocytes. The amount of ^{125}I-LDL used was also determined to be saturating.. In all experiments, controls were done consisting of ^{125}I-LDL alone and ^{125}I-LDL plus anti-LDL. For controls of FcγR specificity, the binding of ^{125}I-LDL in parallel experiments was done in the presence of excess amounts of the corresponding anti-FcγR mAb. For controls of LDL specificity, in some experiments the binding of ^{125}I-LDL to cells treated with bispecific antibodies was done in presence of excess (1 mg/ml) unlabeled LDL. A typical experiment is shown in Fig. 1. For monocytes (Fig. 1A), relative to no antibody and anti-LDL antibody controls: anti-FcγRI x anti-LDL bound five times as much ^{125}I-LDL; anti-FcγRII x anti-LDL bound twenty times as much ^{125}I-LDL; and anti-HLA Class I x anti-LDL bound eight times as much ^{125}I-LDL. The unconjugated anti-LDL bound no ^{125}I-LDL to cells because murine IgG1 mAb do not bind with high affinity to any type of human FcγR (Looney, R.J., et al. (1986) J. Immunol. 136:1641-1647; Lubeck, M.D., et al. (1985) J. Immunol. 135:1299-1304). Using PMN (Fig. 1B), the amount of anti-FcγRIII x anti-LDL bound was twenty times the controls. When binding of heteroantibodies was done in the presence of an excess amount of the corresponding anti-FcγR or anti-HLA Class I mAb, the subsequent binding of ^{125}I-LDL was reduced to control levels (hatched bars). As was also expected, binding of ^{125}I-

LDL to cells labeled with heteroantibodies was totally inhibited in the presence of excess unlabeled LDL.

Effects of bispecific LDL*-IC

A typical experiment of the effects of bispecific LDL*-IC on monocytes is shown in Fig. 2. Monocyte were treated with preformed bispecific LDL*-IC (see "Methods"), prepared by preincubating BODIPY-LDL with varying amounts of bispecific anti-FcγRI x anti-LDL, anti-FcγRII x anti-LDL, and as a non-FcγR control, anti-HLA Class I x anti-LDL. The uptake of LDL* in the absence and presence of unconjugated anti-LDL was minimal and equal to that mediated by anti-HLA Class I x anti-LDL, and did not change with increasing concentrations of antibody or heteroantibody, respectively (Fig. 2A). This occurred despite the fact that the anti-HLA Class I bispecific was effective in delivering LDL to cells (Fig. 1A). However, treatment with bispecific LDL*-IC prepared with anti-FcγRI x anti-LDL or anti-FcγRII x anti-LDL resulted in an approximately 2- and 3.5-fold higher level respectively, of cell-associated LDL* compared with controls. Moreover, the highest level of cell-associated LDL* peaked at 1 μg/ml of heteroantibody. Relative to the LDL* alone control, more than 90% of the cells were fluorescent, and the distribution of fluorescence was unimodal. For several similar experiments the response to bispecific LDL*-IC relative to LDL-alone controls was usually two-fold for anti-FcγRI x anti-LDL, and three to seven-fold for anti-FcγRII x anti-LDL. The inability of the anti-HLA Class I x anti-LDL to mediate uptake of LDL* suggests that myeloid cell FcγR may interact uniquely with bispecific LDL*-IC and is consistent with previous studies showing that FcγR were the only phagocytic or cytotoxic trigger molecules on myeloid cells (Fanger, M.W., et al. (1989) Immunology Today 10:92-99). Unconjugated anti-LDL did not cause uptake of LDL* because as stated above, murine IgG1 mAb do not bind with high affinity to any type of FcγR (Looney, R.J., et al., supra; Lubeck, M.D., et al., supra). The precipitin curve-like nature of the response to bispecific LDL*-IC is consistent with the interpretation that these molecules are reacting with multiple epitopes on the LDL molecules that enhance the ability of the complexes to bind and trigger metabolic uptake. Similar results with such molecules have also been reported in other systems (Taylor, R.P., et al. (1991) Proc. Natl. Acad. Sci. USA 88:3305-3309). For experiments described below where the amount of heteroantibody was not varied, the concentration of heteroantibody used was taken from the peaks of curves shown in Fig. 2.

Effects of temperature on the response to bispecific LDL*-IC

In order to establish that bispecific LDL*-IC were undergoing metabolic uptake by monocytes, we studied the effects of temperature on this phenomenon. In a typical experiment monocytes (Fig. 2B) were treated for 18 hours with bispecific LDL*-IC

prepared with anti-FcγRII x anti-LDL at both 4°C and 37°C. Cell-associated fluorescence of LDL*-IC bound at 4°C was low, relative to the total amount that had accumulated at 37°C. Despite the fact that bispecific LDL*-IC binds significantly to cells in the cold (Fig. 1), there was little change in cell-associated LDL* with time of incubation at 4°C. At 37°C however, the accumulation of LDL*-IC was 4.4-times that of LDL*-alone. As phagocytic processes are inhibited at 4°C (see kinetic and degradation studies below), these data are consistent with the interpretation that at 37°C bispecific LDL*-IC were undergoing metabolic uptake. Also, since the effects of bispecific LDL*-IC at 4°C or 37°C are completely inhibited in the presence of excess anti-FcγR mAb (Fig. 1), it is likely that uptake occurs through specific FcγR and not through nonspecific pinocytotic pathways.

Kinetic studies

To test whether the kinetics of the response to bispecific LDL*-IC were consistent with metabolic uptake, monocytes were treated with bispecific LDL*-IC prepared with anti-FcγRII x anti-LDL for 2, 4, 12 and 24 hours at 37°C. As shown in Fig. 3, significant amounts of cell-associated LDL* were present after 2 hours of treatment with bispecific LDL*-IC and this continued to increase up to 24 hours. By 24 hours of treatment, the increase relative to cells treated with LDL*-alone was 4.3-fold, consistent with the studies discussed above. The same results were obtained in two other studies. As binding of bispecific LDL*-IC to cells is complete within one hour, the cumulative nature of the response to bispecific LDL*-IC is consistent with metabolic uptake.

Effects of excess LDL and AcLDL on the response to bispecific LDL*-IC

Several experiments were done with monocytes in order to determine if native LDL or scavenger receptors (Fogelman, A.M., et al. (1981) J. Lipid. Res. 22:1131-1141; Knight, B.L. and Soutar, A.K. (1982) Eur. J. Biochem. 125:407-413) were involved in the uptake of bispecific LDL*-IC. In preliminary experiments, preparations of unlabeled LDL or AcLDL were checked for their ability to inhibit uptake of saturating amounts of their BODIPY-conjugates by monocytes at 37°C. LDL at 400 μg/ml, and AcLDL at 75 μg/ml caused greater than 80% inhibition of uptake of their respective BODIPY-conjugates. Thus, those concentrations were used to test for inhibition of uptake of bispecific LDL*-IC by monocytes at 37°C. In a typical experiment (Table 1), pretreatment of monocytes with LDL did not inhibit the uptake of bispecific LDL*-IC (8.4 times LDL* alone), nor did pretreatment with unlabeled AcLDL (6.2 times LDL* alone). Thus a significant response to bispecific LDL*-IC occurred when LDL receptors were blocked with LDL, or when scavenger receptors were blocked with AcLDL. Consistent with the absence of a role for scavenger receptors is the fact that the

antioxidant butylated hydroxytoluene (BHT) or superoxide dismutase had no effect on the response to bispecific LDL*-IC.

TABLE I. Uptake of LDL* or bispecific LDL*-IC in the presence of unlabeled LDL or unlabeled AcLDL.

Condition	Cell-associated fluorescence of LDL* or AcLDL
LDL* plus LDL	25±2
LDL*-IC plus LDL	209±5
LDL* plus AcLDL	51+2
LDL*-IC plus AcLDL	315±3

Monocytes in serum-free medium were treated for 20 hours with LDL* alone or bispecific LDL*-IC consisting of 0.5 μg/ml of anti-FcγRII x anti-LDL plus 1.33 μg/ml of BODIPY-LDL, in the presence of 400 μg/ml unlabeled LDL, or 75 μg/ml of unlabeled AcLDL. Cell-associated LDL* was analyzed by flow cytometry as described in "Methods. Shown are the means ± SD of triplicate measurements of cell-associated LDL* from a representative experiment.

LDL degradation mediated through FcγR

To determine whether or not uptake of bispecific LDL-IC was associated with metabolic degradation of LDL, monocytes were treated with saturating amounts of ^{125}I-LDL plus anti-FcγRI x anti-LDL, anti-FcγRII x anti-LDL, or anti-FcγRIII x anti-LDL as described in "Methods" and incubated for four hours at 37°C. Controls were done consisting of ^{125}I-LDL alone, and ^{125}I-LDL plus anti-LDL, both with and without cells. Cell-free degradation of ^{125}I-LDL with and without antibody were approximately equal; therefore all cell-free control values were averaged to correct for specific degradation mediated with bispecifics. The results of three experiments are summarized in Fig. 4. After correction for cell-free degradation, treatment of monocytes with each type of heteroantibody resulted in degradation of ^{125}I-LDL that appeared to correlate with the levels of expression of the respective type of FcγR. The most degradation was seen in association with anti-FcγRII x anti-LDL and was significant when compared to both of the controls (4.2-times No Ab and 9.5 times anti-LDL, both p = .001). Degradation associated with anti-FcγRI x anti-LDL was more than half of that associated

with anti-FcγRII x anti-LDL and was also significant when compared to both controls (2.6 times No Ab, p = .005; 5.9 times anti-LDL, p = .001). Although freshly isolated monocytes express relatively few binding sites for FcγRIII, LDL degradation was also associated with bispecific anti-FcγRIII x anti-LDL (3.2 times anti-LDL, p = .05).

Metabolic Processing of LDL via FcγR

To determine the metabolic fate of LDL taken up by monocytes via each Fcγ receptor, monocytes obtained from a one individual were treated with saturating amounts of aggregated LDL (an insoluble preparation prepared by vortex aggregation of LDL for 60 seconds) opsonized with anti-FcγRI x anti-LDL, anti-FcγRII x anti-LDL, or anti-Fcγ RIII x anti-LDL as described in "Methods" and incubated for 24 hours at 37°C. Controls were done consisting of LDL added alone and anti-LDL Fab'_2 opsonized LDL. Cells were then harvested and the lipid portion was extracted into chloroform, dried into nitrogen and reconstituted into 2-propanol. The amount of cellular cholesterol and cholesteryl esters in the resulting lipid portion was analyzed by reverse-phase high performance liquid chromatography (HPLC) substantially according to the method of Araki et al. (Araki, N. et al. (1990) Analytical Biochemistry 185:339-345), the contents of which are incorporated herein by reference. Briefly, following cell harvesting, chloroform /methanol (2/1 v/v) was added and cellular lipids extracted by a modification of the method of Brown et al. ((1979) J.Cell. Biol. 82:597-613). After overnight extraction at 4°C , an organic phase was removed and the aqueous phase was rinsed briefly with 1.0ml of chloroform. The organic phases were combined and dried by a stream of N_2 gas. Lipid extracts were redissolved in 2-propanol for use in HPLC analyses with a Hewlett Packard liquid chromatograph system and a C_8 (3.9mm x 15cm, 5µm, 300Å) reverse phase HPLC column (Waters Associate, USA).

The results of the HPLC analyses on cellular cholesterol and cholesteryl ester mass retained by monocytes following treatment with each type of heteroantibody (i.e., anti-FcγRI x anti-LDL, anti-FcγRII x anti-LDL, and anti-FcγRIII x anti-LDL) indicated that, in cells obtained from at least one individual, a significantly reduced amount of lipid (mg/ml cell protein) was retained by the cells treated with anti-FcγRI x anti-LDL opsonized LDL compared to the other heteroantibodies. Thus, targeting LDL to FcγRI with a bispecific molecule may result in decreased retention of lipids by the cell.

DISCUSSION

The data suggests that the effects of bispecific LDL*-IC occur independently of native LDL receptors or scavenger receptors that are blocked by AcLDL. As previously discussed, effects of bispecific LDL*-IC were completely inhibited only by pretreatment of cells with excess anti-FcγR mAb (Fig. 1), and are thus dependent upon binding to Fcγ R. As the uptake of bispecific LDL*-IC relative to LDL* alone was not inhibited by

pretreatment of cells with excess unlabeled LDL or AcLDL (Fig. 4), it is unlikely that Fc γR functioned only to fix the complex to the cell surface thereby allowing LDL* to enter via native LDL receptors or scavenger receptors.

It is unlikely for several reasons that treatment of cells with bispecific LDL*-IC caused superoxide production that resulted in LDL* oxidation and subsequent interaction with scavenger receptors (Cathcart, M.K., et al. (1985) J. Leukocyte Biol. 38:341-350; Cathcart, M.K., et al. (1989) J. Immunol. 142:1963-1969). Most importantly, the presence of antioxidants had no effect on the uptake of bispecific LDL*-IC over a twenty hour period. The fact that the response to bispecific LDL*-IC was significant by two to four hours of stimulation (Fig. 3) also argues against a major influence of superoxide-induced modification of LDL at least at those time points, as others have shown using human monocytes, that LDL oxidation triggered by superoxide production began six hours after activation with immune complexes (Cathcart, M.K., et al. (1989) J. Immunol. 142:1963-1969. Moreover, since blockade of scavenger receptors with AcLDL failed to inhibit the response to bispecific LDL*-IC, it is difficult to envision that superoxide anion-induced modification of LDL* was contributing in a major way in these studies.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. A bispecific molecule comprising a first binding specificity for human low density lipoprotein (LDL) or fragment thereof, and a second binding specificity for an Fcγ receptor for immunoglobulin G on a human effector cell.

2. A bispecific molecule of claim 1 which binds to the Fcγ receptor without being blocked by the binding of monomeric immunoglobulin G thereto.

3. A bispecific molecule of claim 2, wherein said second binding specificity is for an Fcγ receptor selected from the group consisting of FcγRI receptor, FcγRII receptor and FcγRIII receptor.

4. A bispecific molecule of claim 2, wherein said second binding specificity is provided by an antibody which immunologically binds an Fcγ receptor selected from the group consisting of FcγRI receptor, FcγRII receptor and FcγRIII receptor.

5. A bispecific molecule of claim 4, wherein said antibody immunologically binds the FcγRI receptor.

6. A bispecific molecule of claim 4, wherein said antibody immunologically binds the FcγRII receptor.

7. A bispecific molecule of claim 4, wherein said antibody immunologically binds the FcγRIII receptor.

8. A bispecific molecule of claim 4, wherein said first binding specificity for human LDL or fragment thereof is provided by an LDL-specific antibody or antigen-binding fragment thereof.

9. A bispecific molecule of claim 8 which is a bispecific antibody.

10. A bispecific molecule of claim 8 which is a heteroantibody.

11. A heteroantibody comprising a first antibody or antigen binding fragment thereof which immunologically binds human low density lipoprotein (LDL), and a second antibody or antigen binding fragment thereof which immunologically binds an Fc γ receptor for immunoglobulin G on a human effector cell, wherein said heteroantibody binds to said Fcγ receptor without being blocked by the binding of immunoglobulin G thereto.

12. A heteroantibody of claim 11, wherein said second antibody binds an Fcγ receptor selected from the group consisting of FcγRI receptor, FcγRII receptor and Fcγ RIII receptor.

13. A heteroantibody of claim 11, comprising a Fab fragment which immunologically binds human LDL and a Fab fragment which immunologically binds an Fcγ receptor selected from the group consisting of FcγRI receptor, FcγRII receptor and FcγRIII receptor.

14. A target-specific effector cell, comprising an effector cell expressing an Fcγ receptor for the Fc portion of immunoglobulin G, and a bispecific molecule bound to the Fcγ receptor, said bispecific molecule comprising at least one binding specificity for human low density lipoprotein (LDL) or a fragment thereof and at least one binding specificity for the Fcγ receptor, wherein the bispecific molecule binds to the Fcγ receptor without being blocked by binding of monomeric immunoglobulin G thereto.

15. A target-specific effector cell of claim 14, wherein said effector cell is selected from the group consisting of a human monocyte, a macrophage, a tissue macrophage and a polymorphonuclear cell.

16. A target-specific effector cell of claim 14, wherein said bispecific molecule is a heteroantibody comprising a first antibody or antigen binding fragment thereof which immunologically binds human low density lipoprotein (LDL), and a second antibody or antigen binding fragment thereof which immunologically binds an Fc γ receptor selected from the group consisting of FcγRI receptor, FcγRII receptor and Fcγ RIII receptor.

17. A method of treating atherosclerosis in an individual by reducing low density lipoprotein (LDL) levels in the individual, comprising administering to the individual a therapeutic amount of a bispecific molecule, said bispecific molecule comprising:

(a) at least one first binding specificity for human low density lipoprotein (LDL), or a fragment thereof; and

(b) at least one second binding specificity for an Fcγ receptor for immunoglobulin G on a human effector cell, wherein said bispecific molecule binds the Fcγ receptor without being blocked by binding of monmeric immunoglobulin G thereto.

18. A method of claim 17, wherein the at least one first binding specificity is provided by a human LDL-specific antibody or antigen-binding fragment thereof.

19. A method of claim 17, wherein the at least one second binding specificity is provided by an antibody or antigen-binding fragment which immunologically binds an Fcγ receptor selected from the group consisting of FcγRI receptor, FcγRII receptor and FcγRIII receptor.

20. A method of claim 17, wherein the bispecific molecule is a heteroantibody comprising a first antibody or antigen-binding fragment thereof which immunologically binds human low density lipoprotein (LDL), and a second antibody or antigen-binding fragment thereof which immunologically binds an Fcγ receptor selected from the group consisting of FcγRI receptor, FcγRII receptor and FcγRIII receptor.

21. A method of claim 20, wherein the heteroantibody comprises a Fab fragment which immunologically binds human LDL and a Fab fragment which immunologically binds an an Fcγ receptor selected from the group consisting of FcγRI receptor, FcγRII receptor and FcγRIII receptor.

22. A method of claim 17, further comprising administering to the individual a cytokine.

23. A method of determining the ability of a human white blood cell to develop into a cholesteryl ester-laden foam cell to thereby identify an individual as at risk of developing atherosclerosis, the method comprising:

(a) obtaining white blood cells from the individual;

(b) contacting the white blood cells with an amount of human low density lipoprotein (LDL) and an amount of a heteroantibody comprising (i) a first antibody or antigen-binding fragment thereof which immunologically binds human LDL, and (ii) a second antibody or antigen-binding fragment thereof which immunologically binds an Fcγ receptor selected from the group consisting of FcγRI receptor, FcγRII receptor and FcγRIII receptor, under conditions appropriate for binding of the heteroantibody to the human LDL and the Fcγ receptor of the white blood cell; and

(c) determining the level of cholesteryl ester accumulation in the cell as indicative of the tendency of the cell to develop into a foam cell.

24. A method of claim 23, wherein the white blood cell is a monocyte or macrophage.

FIG. 1A

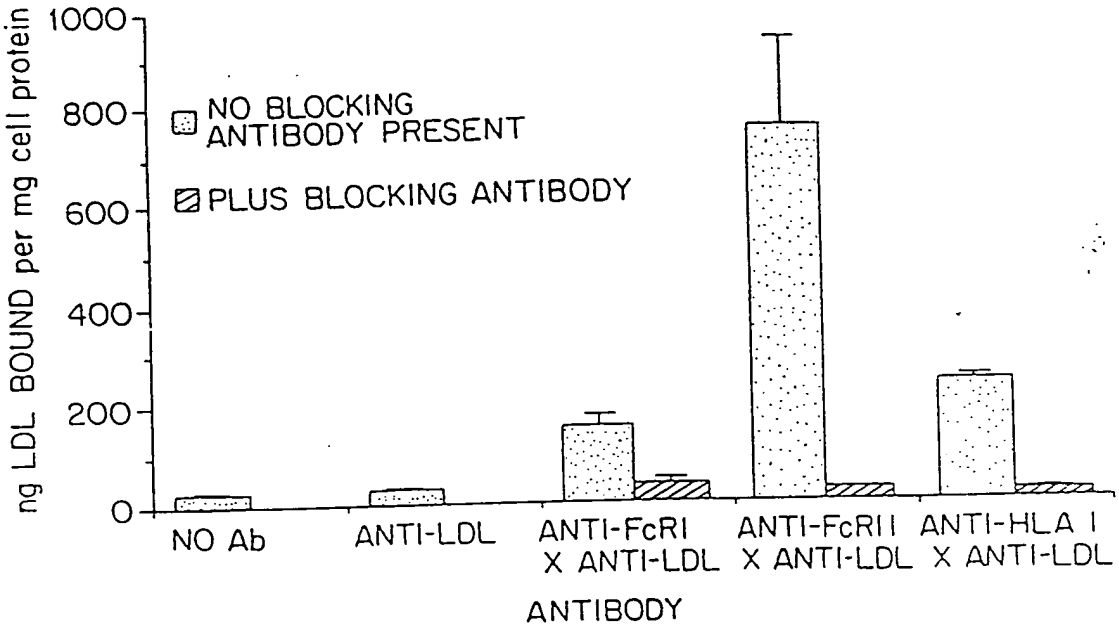

ng LDL BOUND per mg cell protein
1000
800
600
400
200
0
NO BLOCKING ANTIBODY PRESENT
PLUS BLOCKING ANTIBODY
NO Ab
ANTI-LDL
ANTI-FcRI X ANTI-LDL
ANTI-FcRII X ANTI-LDL
ANTI-HLA I X ANTI-LDL
ANTIBODY

FIG. 1B

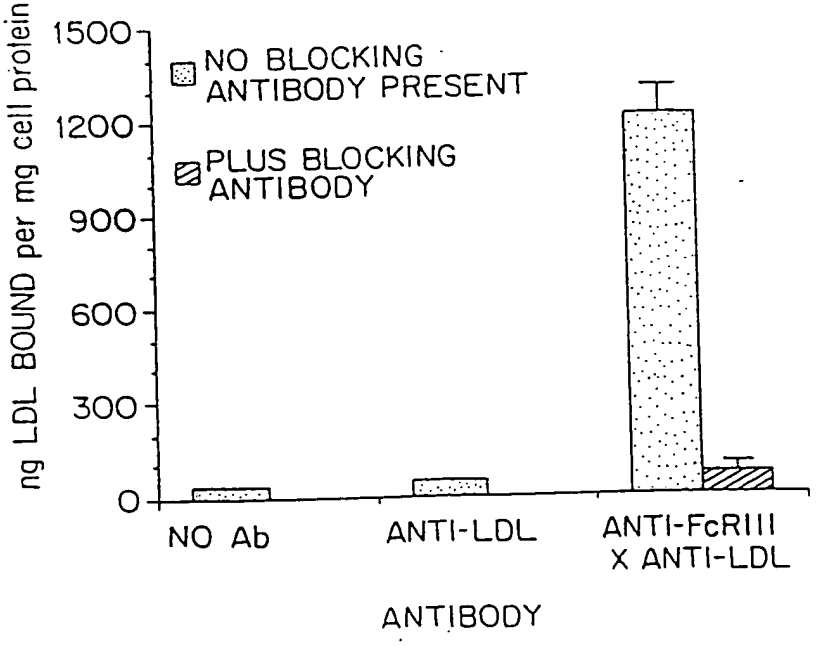

ng LDL BOUND per mg cell protein
1500
1200
900
600
300
0
NO BLOCKING ANTIBODY PRESENT
PLUS BLOCKING ANTIBODY
NO Ab
ANTI-LDL
ANTI-FcRIII X ANTI-LDL
ANTIBODY

FIG. 2A

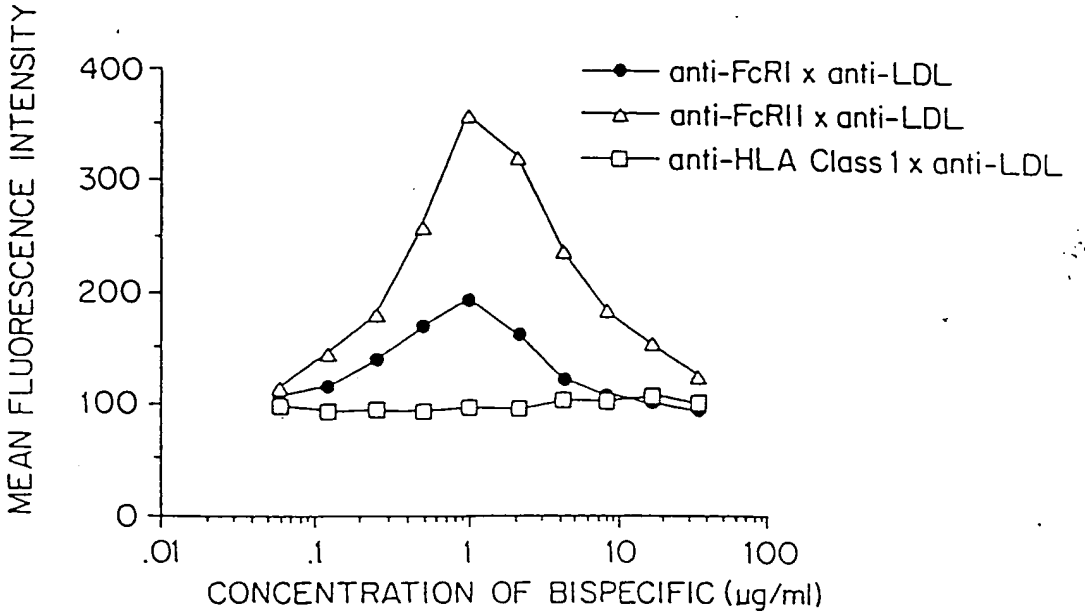

MEAN FLUORESCENCE INTENSITY
400
300
200
100
0
.01
.1
1
10
100
CONCENTRATION OF BISPECIFIC (µg/ml)
anti-FcRI x anti-LDL
anti-FcRII x anti-LDL
anti-HLA Class 1 x anti-LDL

FIG. 2B

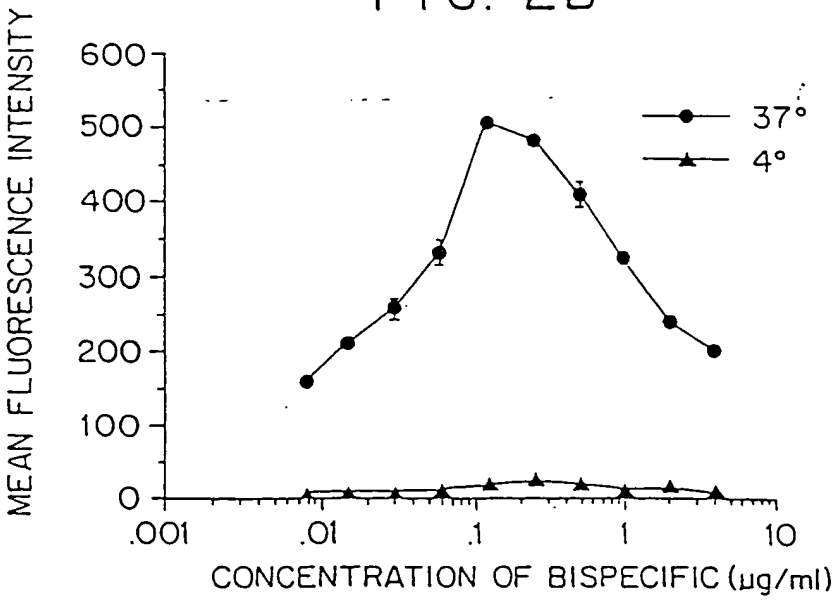

MEAN FLUORESCENCE INTENSITY
600
500
400
300
200
100
0
.001
.01
.1
1
10
CONCENTRATION OF BISPECIFIC (µg/ml)
37°
4°

FIG. 3

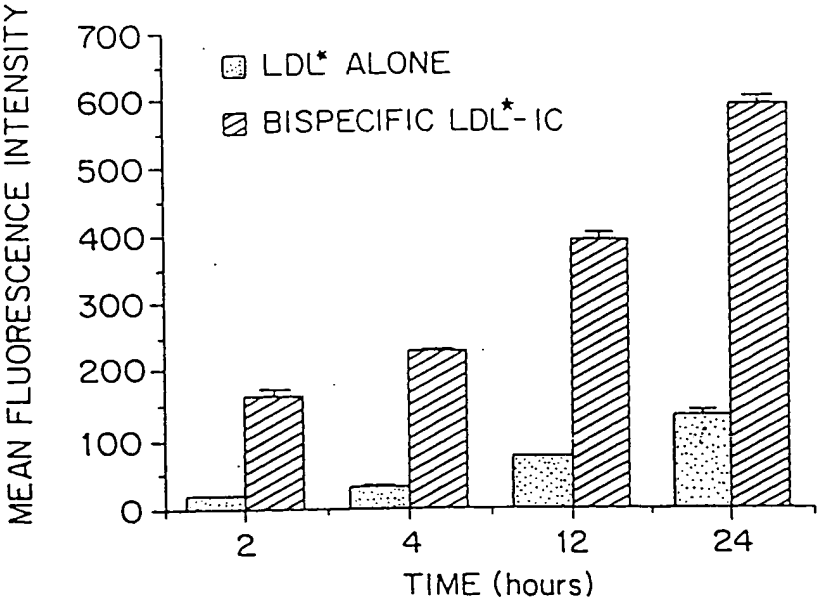
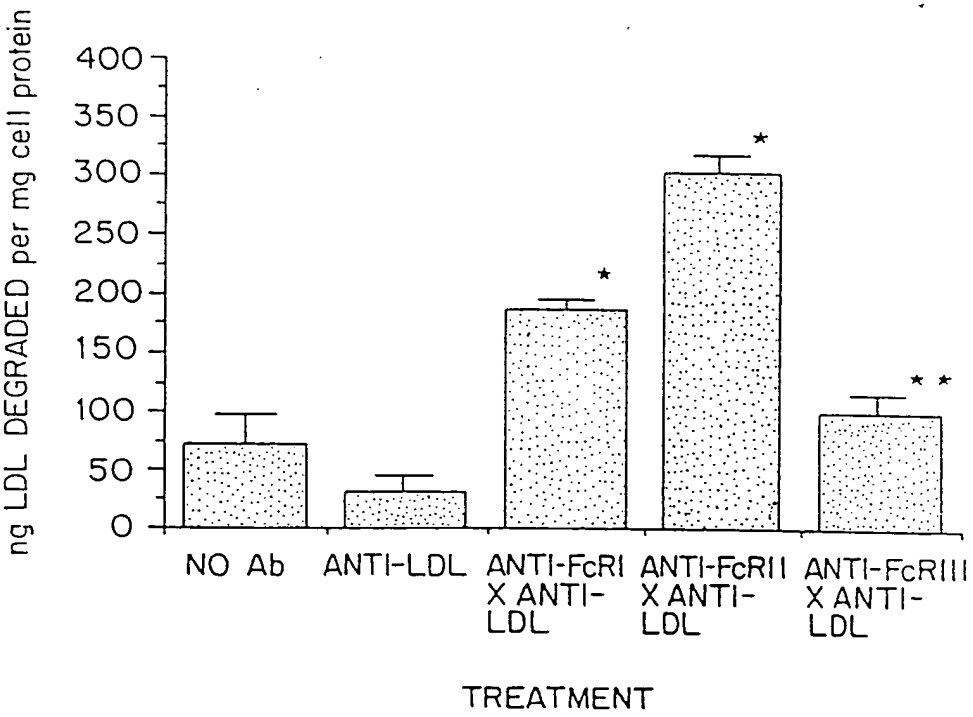

MEAN FLUORESCENCE INTENSITY
700
600
500
400
300
200
100
0
LDL* ALONE
BISPECIFIC LDL*-IC
2
4
12
24
TIME (hours)

FIG. 4


ng LDL DEGRADED per mg cell protein
400
350
300
250
200
150
100
50
0
*
*
* *
NO Ab
ANTI-LDL
ANTI-FcRI X ANTI-LDL
ANTI-FcRII X ANTI-LDL
ANTI-FcRIII X ANTI-LDL
TREATMENT

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 93/09556

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12P21/08 C12N5/08 A61K39/395 A61K37/02 G01N33/577
G01N33/92

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12P C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE JOURNAL OF IMMUNOLOGICAL METHODS vol. 129, no. 2 , 25 May 1990 , AMSTERDAM, THE NETHERLANDS pages 277 - 282 L. KARAWAJEW ET AL. 'Flow sorting of hybrid hybridomas using the DNA stain Hoechst 33342.' see abstract ---	1-24
Y	WO,A,91 05871 (MEDAREX, INC.) 2 May 1991 see claims --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed.

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 January 1994	22 -02- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA vol. 88, no. 21 , 1 November 1991 , WASHINGTON DC, USA pages 9593 - 9597 R. CONNOR ET AL. 'Fc receptors for IgG (FcgammaRs) on human monocytes and macrophages are not infectivity-receptors for human immunodeficiency virus type 1 (HIV-1): Studies using bispecific antibodies to target HIV-1 to various myeloid cell surface molecules, including the FcgammaR.' see abstract	1-24
A	WO,A,92 05793 (MEDAREX, INC.) 16 April 1992 see claims	1-24
P,X	ARTERIOSCLEROSIS AND THROMBOSIS vol. 12, no. 10 , October 1992 , DALLAS TX, USA pages 1131 - 1138 P. MORGANELLI ET AL. 'Redirected targeting of LDL to human monocyte Fcgamma receptors with bispecific antibodies.' . see the whole document	1-24
P,X	CRITICAL REVIEWS ON IMMUNOLOGY vol. 12, no. 3-4 , 1992 , BOCA RATON FL, USA pages 101 - 124 M. FANGER ET AL. 'Bispecific antibodies.' see the whole document	1-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09556

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 17-22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects ofthe compound/composition:

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/US 93/09556

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9105871	02-05-91	CA-A-	2069960	21-04-91
WO-A-9205793	16-04-92	AU-A-	8869491	28-04-92
		CA-A-	2093022	06-04-92
		EP-A-	0553244	04-08-93